

## Original Article

# A therapeutic vaccine targeting HPV E6/E7 with intrinsic Toll-like receptor 2 agonist activity induces antitumor immunity

Yuh-Pyng Sher<sup>1,2,3,4</sup>, Chi Lee<sup>5</sup>, Shin-Yu Liu<sup>5</sup>, I-Hua Chen<sup>5</sup>, Ming-Hui Lee<sup>5</sup>, Fang-Feng Chiu<sup>5</sup>, Chih-Hsiang Leng<sup>1,5,6</sup>, Shih-Jen Liu<sup>1,5,6</sup>

<sup>1</sup>Graduate Institute of Biomedical Sciences, <sup>2</sup>Chinese Medicine Research Center, <sup>3</sup>Research Center for Chinese Herbal Medicine, China Medical University, Taichung, Taiwan; <sup>4</sup>Center for Molecular Medicine, China Medical University Hospital, Taichung 404, Taiwan; <sup>5</sup>National Institute Infectious Diseases and Vaccinology, National Health Research Institutes, Miaoli, Taiwan; <sup>6</sup>Graduate Institute of Medicine, Kaohsiung Medical University, Kaohsiung, Taiwan

Received November 8, 2018; Accepted November 15, 2018; Epub December 1, 2018; Published December 15, 2018

**Abstract:** The E6 and E7 oncoproteins of human papillomavirus (HPV) are ideal targets for developing immunotherapeutic approaches to treat HPV-associated tumors. Our previous studies showed that a recombinant lipidated HPV16 E7 mutant (rlipo-E7m) with inactivation of the E7 oncogenic functions can activate antigen presenting cells through Toll-like receptor 2 (TLR2) and induce antitumor immunity. Given that some HPV-associated tumors overexpress E6 but not E7, it is necessary to include therapeutic agents containing HPV E6 in therapeutic vaccine development to broaden the utility of the vaccine. In this study, we further incorporated a mutant HPV16 E6 (E6m) into rlipo-E7m to generate rlipo-E6mE7m, which could elicit both E6- and E7-specific immune responses after immunization. The rlipo-E6mE7m immunization induced higher levels of T cell proliferation and cytotoxic T lymphocyte response than the nonlipidated recombinant E6mE7m (rE6mE7m) immunization. Accordingly, a single-dose administration of rlipo-E6mE7m at day 7 after tumor inoculation in mice showed complete inhibition of tumor growth, whereas administration of rE6mE7m did not. These results demonstrated that rlipo-E6mE7m could be used in tumors with E6 and/or E7 expression via the induction of E6- and E7-specific immunity.

**Keywords:** Lipoprotein, TLR2, human papillomavirus, E6, E7

## Introduction

Human papillomavirus (HPV) infection is the main risk factor for the development of several cancers, such as head and neck cancer and cervical cancer [1]. The number of newly diagnosed cervical cancer cases per year worldwide is over 600,000 [2]. Although prophylactic HPV vaccination to prevent HPV infection has been applied in health care and is expected to reduce the incidence of HPV-mediated cancers [3], cervical cancer is still the leading cause of cancer-related death in women because of the lack of therapeutic vaccines to treat patients with developed tumors. Therefore, therapeutic medicine to efficiently reduce cervical cancer development is an unmet medical need that could decrease cancer mortality.

Currently, the two major viral oncoproteins, E6 and E7, are considered targets for developing therapeutic vaccines because they drive cellular immortalization and maintain the transformed phenotype during tumor progression. They exert functions by binding with many cellular proteins to activate cancer hallmarks [4, 5]. For example, E6 and E7 can interact with the pro-apoptotic proteins p53 and retinoblastoma tumor suppressor protein (pRB), respectively, to promote the targeted degradation of proteins with different crucial roles in tumorigenesis [6]. Moreover, the joint functioning of the E6 and E7 oncoproteins is required from early to later stages of HPV-induced malignancy [7]. In addition, the sequences of both proteins are well-conserved across a broad range of HPV subtypes [8, 9]. Thus, the two oncoproteins are

## A recombinant lipidated HPV E6 and E7 fusion protein induces antitumor immunity

considered to be potential targets for therapeutic interventions using vaccines against HPV-associated tumors.

Different forms of HPV therapeutic vaccines have been developed and tested in preclinical and clinical trials, such as peptide- and protein-based vaccines [10]. Although these kinds of vaccines are safe, stable, and easy to manufacture, adjuvants and immunostimulatory molecules are needed to effectively elicit dendritic cell (DC) and T cell functions. In other words, a vaccine candidate with intrinsic adjuvant activity would be the best design.

Bacterial lipoproteins, a set of membrane proteins with lipid modifications, have been shown to play key roles in adhesion to host cells, modulation of inflammatory processes, and virulence-associated functions [11]. Several of the lipoproteins, such as lipidated outer surface protein A (OspA) of *Borrelia burgdorferi* [13], outer membrane lipoprotein I (OprI) from *Pseudomonas aeruginosa* [14], and the 19-kDa antigen of *M. tuberculosis* [15], have been identified as potential vaccine components that could induce proinflammatory effects and immune activation [12]. It has been demonstrated that the use of lipoproteins is a superior approach for the development of prophylactic vaccines against infectious diseases. Recently, we developed a platform for producing a high yield of recombinant lipoproteins [16] and proved that the lipid moiety of the lipo-immunogen, derived from bacterial lipoproteins, can be recognized as a danger signal to induce both innate and adaptive immunity in animals receiving lipoprotein vaccinations [17, 18]. Moreover, we demonstrated that recombinant lipo-immunogen can induce higher virus-neutralizing antibody responses than the nonlipid form [16].

Continued expression of the two HPV oncoproteins is required to maintain the malignant phenotype; however, both E6 and E7 can individually transform primary cells through interacting with different cellular targets, and their major roles in tumorigenesis are different. In cervical tissue, E7 appears to serve as the dominant oncogene to produce the early stages of reproductive tract carcinomas, whereas E6 promotes the later stage of tumor progression [19]. A similar effect of the dominant oncogene E7 was detected in transgenic models of head and neck cancer [20]. In our previous study, we

demonstrated that a recombinant lipidated HPV E7 mutant (rlipo-E7m) can provide protective immunity against cervical cancer in a mouse model [21]. Since the E6 and E7 oncoproteins are dominant at different stages during tumor development, targeting both the E6 and E7 oncoproteins would be a promising approach for the development of therapeutic vaccines that could provide comprehensive therapeutic effects in HPV-mediated cancers.

### Materials and methods

#### *Cloning and expression of recombinant protein rE6mE7m and rlipo-E6mE7m*

The synthetic nucleotide sequence of the HPV16 E6mE7m gene in the pBR322 vector with added Nde I and Xho I restriction enzyme sites flanking the genes at the 5'- and 3'-ends, respectively, were purchased from Invitrogen (Carlsbad, CA, USA). The gene fragments were cloned into the Nde I-Xho I sites of the expression vector pET-22b(+) (Novagen, Madison, WI) to generate the plasmids pET-22b E6mE7m and pET-22b A23E6mE7m. An additional hexahistidine tag (HisTag) sequence was inserted at the C-terminal end of each of the recombinant proteins.

For the production of recombinant E6mE7m (rE6mE7m), the expression plasmid pET-22b E6mE7m was transformed into the *E. coli* strain BL21 Star (DE3) (Invitrogen, CA, USA). The transformed cells were cultured in LB medium at 37°C and then induced with 1 mM IPTG for 4 hr at O.D.=0.6. The cells were harvested and resuspended in 20 mM Tris-Cl, 500 mM NaCl, 50 mM sucrose, pH 8.0 buffer for further purification.

For the production of rlipo-E6mE7m, plasmid pET-22b A23E6mE7m was transformed into the CD43 (DE3) *E. coli* strain [16]. The transformed cells were cultured in M9 medium at 37°C overnight and then induced with 1 mM IPTG at 12°C for 3 days. The cells were harvested and resuspended in 20 mM Tris-Cl, pH 8.9 buffer for further purification.

#### *Purification of recombinant proteins*

Recombinant E6E7m (rE6E7m) and lipidated E6E7m (rlipo-E6E7m) were purified by disrupting the harvested cells in a French press

## A recombinant lipidated HPV E6 and E7 fusion protein induces antitumor immunity

(Constant Systems, Daventry, UK) at 27 Kpsi in homogenization buffer [500 mM NaCl; 50 mM sucrose; 20 mM Tris-HCl (pH 8.0)]. The cell lysate was clarified by centrifugation (80,000 × g for 40 min). The pellet was solubilized with urea buffer [8 M urea; 50 mM sucrose; 20 mM Tris-HCl (pH 8.0)] and homogenized with a Dounce homogenizer. The mixture was again clarified by centrifugation (80,000 × g for 40 min). The recombinant proteins were extracted in guanidine hydrochloride (GdnHCl) buffer and purified by using a Ni-NTA resin column. The purified proteins were dialyzed against phosphate buffer. The endotoxin levels in the final product were below 0.03 EU/mg.

### *Characterization of the recombinant proteins*

Purified rE6mE7m and rlipo-E6mE7m were detected by an anti-His antibody (Santa Cruz, CA, USA) and a mouse anti-human papillomavirus 16 E7 antibody (VALDOSPAN GmbH, cat VS13004) by Western blotting. To identify the N-terminal fragment of rlipo-E6mE7m, rlipo-E6mE7m was first dialyzed against 25 mM ammonium bicarbonate at pH 8.5 and then treated with trypsin (Promega Co., Madison, WI) at a rlipo-E6mE7m:trypsin ratio of 50:1 (Wt/Wt) in 25 mM ammonium bicarbonate (pH 8.5) for 16 hrs at 37°C. The reaction mixture was further purified using Ziptip (Millipore, Massachusetts). One microliter of the trypsin-digested protein was mixed with 1 µL saturated α-ciano-4-hydrozycinnamic acid solution (Sigma-Aldrich, St. Louis, MO) in 75% acetonitrile/0.1% trifluoroacetic acid. One microliter of the mixture was placed on the target plate of a matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometer (Bruker Daltonics, Germany) for analysis. The results obtained from the MALDI-TOF analysis described above indicated that the partial trypsin digestion products corresponded to the N-terminal fragments of rlipo-E6mE7m, and these peptides were lipidated.

### *Activation of bone marrow-derived dendritic cells (BM-DCs)*

BM-DCs derived from C57BL/6 mice were assessed as previously described [16]. Briefly, mouse bone marrow cells were cultured at a density of  $2 \times 10^6$  cells/ml in petri dishes containing 10 ml complete medium (CM: RPMI

1640 containing 10% FCS, 1% P/S, 50 µM 2-ME, 2 mM glutamine, 25 mM HEPES, and 1 mM sodium pyruvate) with 20 ng/ml recombinant mouse GM-CSF. On day 3, an additional 10 ml CM medium containing 20 ng/ml GM-CSF was added. On day 6, the nonadherent cells were collected and transferred into 24-well plates. BM-DCs ( $1 \times 10^6$  cells/ml) were stimulated with LPS (0.1 µg/ml) or the indicated concentrations of rE6mE7m or rlipo-E6mE7m for 24 hrs. At this time point, the supernatants from the different cultures were collected and stored at -20°C for cytokine assays. The cell surface markers (CD40 and CD80) of BM-DCs were analyzed using flow cytometry (FACSCalibur, BD bioscience, San Jose, CA). The production of cytokines (TNFα) by BM-DCs was determined using ELISA kits (eBioscience, CA, USA).

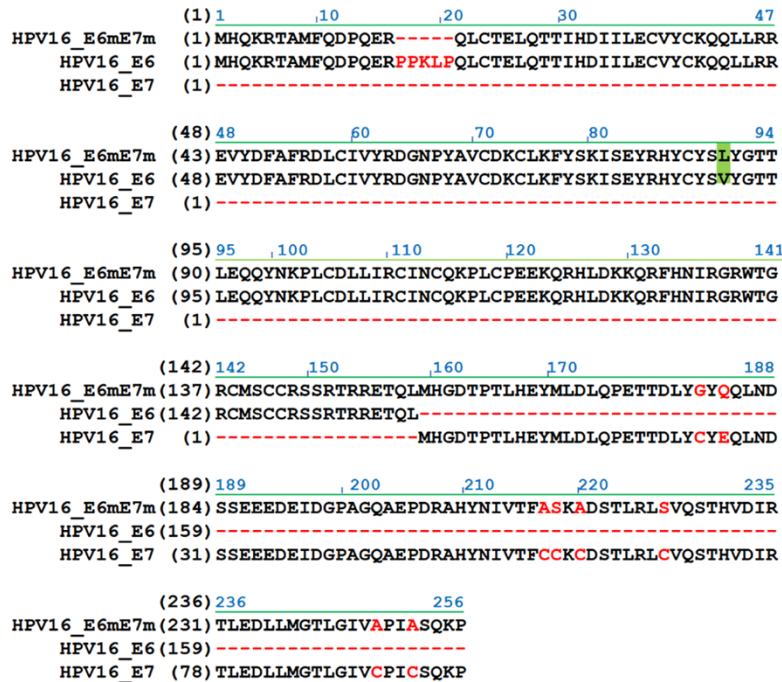
### *Lymphocyte proliferation assay*

Lymphocytes from the spleen and lymph nodes of C57BL/6 mice were plated at a density of  $2 \times 10^5$ /well in flat bottom 96-well plates and stimulated with 10 µg/mL rE6mE7m or rlipo-E6mE7m for 48 hrs at 37°C in the presence of 5% CO<sub>2</sub> in a humidified incubator. In the final 24 hrs of culture, 1 µCi [<sup>3</sup>H]-thymidine (5 µCi/mmol; Amersham, Arlington Heights, IL) was added to each well, and the plates were again incubated overnight in a humidified CO<sub>2</sub> atmosphere at 37°C. Following the incubation, the supernatant was aspirated, and the cells were harvested using a FilterMate (Packard, Meriden, CT) automatic cell harvester. The incorporated radioactivity was determined using a Top Count microplate scintillation counter (Packard, Meriden, CT). All results are presented as the mean cpm ± standard deviation (SD).

### *T cell response assay*

C57BL/6 mice were injected subcutaneously with 10 µg rE6mE7m or rlipo-E6mE7m twice at two-week intervals. Seven days after the second immunization, the splenocytes and lymphocytes from lymph nodes were isolated and restimulated with rE6mE7m or rlipo-E6mE7m (10 µg/ml) for 4-5 days. The supernatants were collected, and the levels of IFN-γ, IL-5, IL-10 or IL-17 were measured by ELISA. The cells were simultaneously assessed by an IFN-γ ELISPOT assay as previously described [21]. Briefly, splenocytes ( $5 \times 10^5$ /well) were added to anti-IFN-γ

# A recombinant lipidated HPV E6 and E7 fusion protein induces antitumor immunity



**Figure 1.** The amino acid sequence of the constructed HPV16 E6mE7m immunogen. E6mE7m proteins were combined with mutated E6 and E7. The mutated sites were highlighted or marked for inactivating the oncogenic activity of E6 and E7. The alignment of E6mE7m and wild-type E6 and E7 was representative. Additionally, a HisTag sequence was included at the C-terminus of the proteins, and the DNA sequence of E6mE7m was modified using the codon usage of *E. coli*. For rlipto-E6mE7m protein expression, the region containing the lipid signal peptide was inserted into the expression plasmid.

antibody-coated plates and cultured in the presence of 10 mg/ml HPV E7-derived peptide RAH (RAHYNIVTF) in a final volume of 200  $\mu$ L CM. Two peptides containing HPV E6 epitopes, KQQ peptides (KQQLLRREYDFAFRDLCIVYRDGN) or EVY peptides (EVYDFAFRDL), were also included. After 48 hrs of incubation, the cells were removed, and the plates were washed with 0.05% (w/v) Tween 20 in PBS. A biotinylated anti-IFN- $\gamma$  antibody was added to each well, and the samples were incubated for 2 hrs. After the detection antibody solution was discarded, a diluted streptavidin-HRP solution was added to the wells for another 1 hr. The spots were developed using 3-amine-9-ethyl carbazole (Sigma-Aldrich, St. Louis, MO) and counted by an ELISPOT reader (Cellular Technology Ltd., Shaker Heights, OH). The data are expressed as the mean  $\pm$  SD of each sample (n=3).

## *In vivo tumor protection experiments*

Six- to twelve-week-old female C57BL/6 mice were subcutaneously inoculated with TC-1 cells

( $2 \times 10^5$ /mouse). After 7 days, the tumor-bearing mice (5-10 mice per group) were injected once with RAH/IFA (10 mg/mouse), rE6mE7m (30 mg/mouse), rlipto-E6mE7m (30 mg/mouse) or PBS. The tumor size was measured at 2-day intervals with calipers in two dimensions, and the tumor volumes were calculated as width<sup>2</sup>  $\times$  length/2. The mice were sacrificed when the tumor size reached 2,000 mm<sup>3</sup> or the mice lost >20% of their initial body weight, in accordance with the Guide for the Institutional Animal Care and Use Committee of NHRI. The protocol was approved by the Institutional Animal Care and Use Committee of NHRI (Permit Number: NHRI-IACUC-103020-A). After the vaccination, the tumor growth was recorded for 60 days.

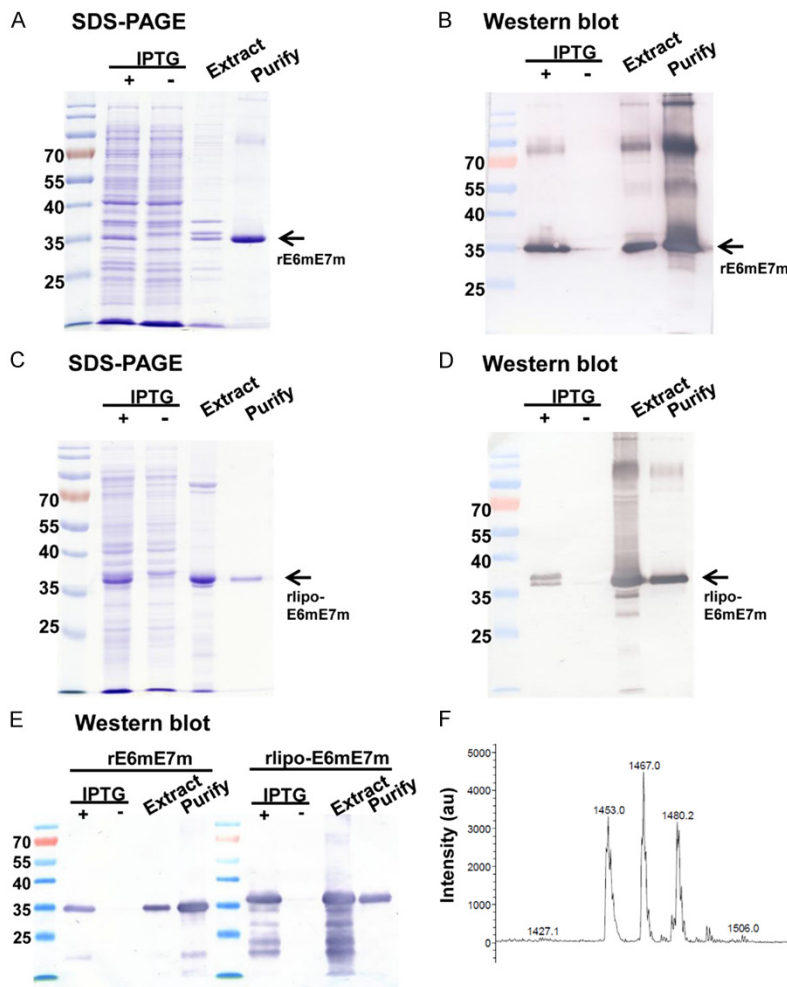
## Results

### *Construction of inactive oncoprotein-HPV E6mE7m immunogens*

To construct the inactive HPV E6mE7m immunogens for vaccination, mutations in the amino acid sequences of E6 and E7 were designed to inactivate their oncogenic functions, and the E6 mutant (E6m) and E7 mutant (E7m) were combined to obtain the fused protein E6mE7m (Figure 1). For the HPV E6 mutant, a deletion of the PRKLP element and a leucine to valine mutation were included to influence the interactions of E6 with E6AP and p53 [22]. For the HPV E7 mutant, we demonstrated that the inactive-E7 immunogen (E7m) still contains the major CTL epitopes [21]. Previously, we identified a fusion sequence (D1, MKKLLIAAMM-AAALAACSQEAKQEVKEAVQAVESDVKDTA) that can be fused with the nonlipidated immunogen to achieve high expression levels of the recombinant lipo-immunogen [16]. To generate rlipto-E6mE7m, a fusion sequence (D1) was inserted at the N-terminus of the E6mE7m gene, and a HisTag was included at the C-terminus of the E6mE7m gene for purification.



## A recombinant lipidated HPV E6 and E7 fusion protein induces antitumor immunity



**Figure 2.** Production and purification of rE6mE7m and rlipo-E6mE7m. (A) The rE6mE7m proteins were monitored in the fraction subjected to IPTG induction and the extracted fraction. The final purified recombinant rE6mE7m proteins were detected using 15% reducing SDS-PAGE followed by Coomassie Blue staining. (B) The rE6mE7m proteins were validated using anti-HisTag antibodies in Western blotting. The nonlipidated form of rE6mE7m was expressed in the *E. coli* BL21 Star (DE3) strain. The arrows indicate the electrophoretic positions of rE6mE7m. (C) The rlipo-E6mE7m protein expression was also monitored by SDS-PAGE and validated using anti-HisTag antibodies in Western blotting (D). The lipidated form of rE6mE7m, rlipo-E6mE7m, was expressed in the *E. coli* BL21 (C43) strain. The arrows indicate the electrophoretic positions of rlipo-E6mE7m. (E) The Western blot analysis of rE6mE7m and rlipo-E6mE7m using an anti-HPV16 E7 antibody is shown. (F) The mass spectrometry analysis of rlipo-E6mE7m is shown. The N-terminal rlipo-E6mE7m fragments were obtained and identified after the digestion of rlipo-E6mE7m with trypsin. The digested sample was analyzed on a Waters® MALDI micro MX™ mass spectrometer. The MALDI-TOF MS spectra revealed the existence of three peaks with  $m/z$  values of 1453, 1467, and 1480.

### Identification and characterization of purified immunogens

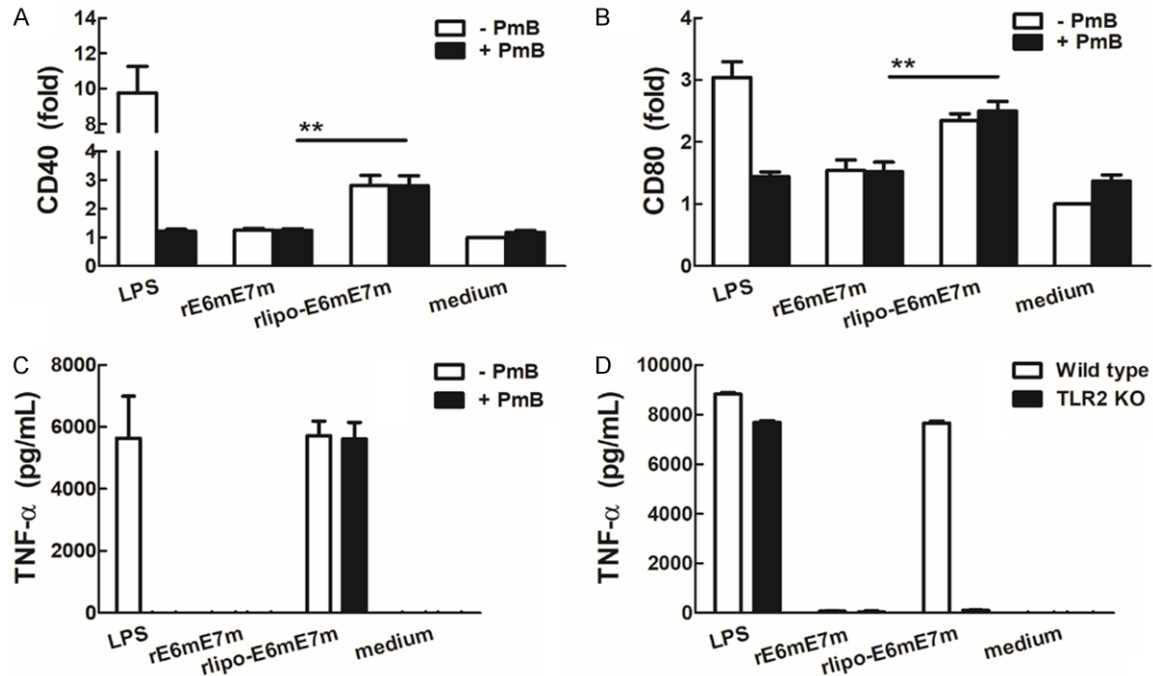
First, the recombinant immunogen rE6mE7m was produced with IPTG induction in the *E. coli* system and then purified using an immobilized

metal affinity chromatography (IMAC) column (Figure 2A). The rE6mE7m proteins were detected by anti-HisTag antibodies (Figure 2B). The rlipo-E6mE7m proteins were produced and purified with a strategy similar to that used for the rE6mE7m proteins (Figure 2C) and were detected by anti-HisTag antibodies (Figure 2D). In addition, the purified rE6mE7m and rlipo-E6mE7m proteins were detected with an anti-HPV E7 antibody (Figure 2E). To identify whether the lipid moiety was added at the N-terminus of the rlipo-E6mE7m proteins, peptide mass fingerprinting (PMF) was performed to measure the major peaks in the mass spectral data derived from rlipo-E6mE7m. After processing the data, three major peaks with masses of 1453, 1467, and 1480.2 Da were identified (Figure 2F). Based on the observation that the distance of the mass between the major peaks was approximately 14 Da, which was the predicted mass of the CH<sub>2</sub> structure from our previous study [21], the spectrum of these major peaks could be considered the signature of the lipid modifications in rlipo-E6mE7m [16].

### rlipo-E6mE7m activates bone marrow-derived dendritic cells through TLR2

To evaluate the function of rlipo-E6mE7m and compare it with that of nonlipidated rE6mE7m, mouse bone marrow-derived DCs (BM-DCs) were used as a model to study the immunostimulatory properties of the immunogens. The lipopolysaccharide (LPS) levels of the purified rE6mE7m and rlipo-E6mE7m proteins were lower than 0.003 EU/μg, and then the

## A recombinant lipidated HPV E6 and E7 fusion protein induces antitumor immunity



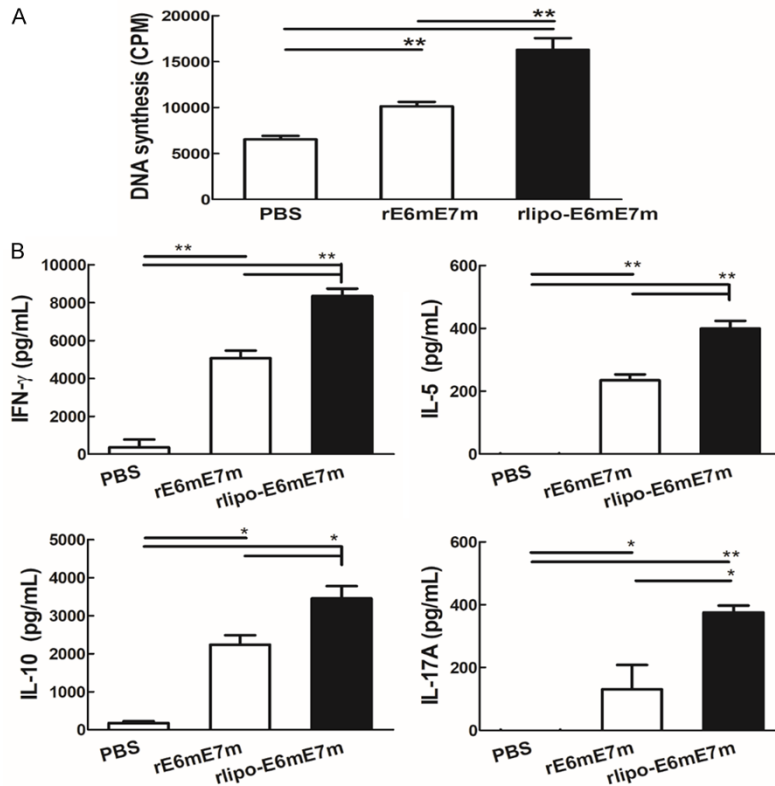
**Figure 3.** ripo-E6mE7m proteins activate bone marrow-derived dendritic cells (BM-DCs). BM-DCs from wild-type mice were cultured in medium supplemented with LPS (0.1  $\mu\text{g}/\text{ml}$ ), rE6mE7m (10  $\mu\text{g}/\text{ml}$ ) or ripo-E6mE7m (10  $\mu\text{g}/\text{ml}$ ) in the presence or absence of polymyxin B (20  $\mu\text{g}/\text{ml}$ ). After a 24-hr incubation, the expression of the dendritic cell surface markers CD40 (A) and CD80 (B) was analyzed using flow cytometry. The experiments were performed in triplicate, and the mean fluorescence intensity (MFI) of the cells cultured in medium alone was defined as 1. (C) TNF- $\alpha$  secretion by dendritic cells was stimulated by ripo-E6mE7m. BM-DCs were cultured in medium supplemented with LPS (0.1  $\mu\text{g}/\text{ml}$ ), rE6mE7m (10  $\mu\text{g}/\text{ml}$ ) or ripo-E6mE7m (10  $\mu\text{g}/\text{ml}$ ) in the presence or absence of polymyxin B (20  $\mu\text{g}/\text{ml}$ ). After a 24-hr incubation, the supernatants were harvested and analyzed for TNF- $\alpha$  production by ELISA. (D) BM-DCs isolated from wild-type and TLR2 knockout (TLR2 KO) mice were cultured in medium supplemented with LPS (0.1  $\mu\text{g}/\text{ml}$ ), rE6mE7m (10  $\mu\text{g}/\text{ml}$ ) or ripo-E6mE7m (10  $\mu\text{g}/\text{ml}$ ), and then TNF- $\alpha$  production was detected by ELISA after a 24-hr incubation.

immunogens were comparatively analyzed for their immunogenicity and efficacy in animal models. Polymyxin B (Pmb), which has been used to clear endotoxin contamination, was added to detect any interference caused by residual LPS. We found that ripo-E6mE7m was capable of stimulating and upregulating the expression of the costimulatory molecules CD40 and CD80 on BM-DCs, while rE6mE7m was ineffective (**Figure 3A** and **3B**). Similar levels of CD40 and CD80 expression on BM-DCs were seen with ripo-E6mE7m stimulation regardless of Pmb addition, indicating that the effects were not caused by residual LPS. Furthermore, with or without Pmb addition, the secretion of TNF- $\alpha$  from BM-DCs was induced by ripo-E6mE7m in a dose-dependent manner, but no TNF- $\alpha$  secretion was detected in the rE6mE7m groups (**Figure 3C**). Moreover, the secretion of TNF- $\alpha$  from BM-DCs was induced by ripo-E6mE7m in wild-type mice but not in

Toll-like receptor 2 knockout (TLR2 KO) mice (**Figure 3D**), indicating that TLR2 is required for the immunostimulatory activity of ripo-E6mE7m. These results indicate that the activation of BM-DCs by ripo-E6mE7m was not due to any residual LPS, and ripo-E6mE7m provided stronger immunostimulatory activity than rE6mE7m. Since previous results have demonstrated that the amino acid sequence of the D1 domain (the fusion partner for recombinant lipoprotein production) is unable to stimulate the production of cytokines [16], these data clearly demonstrated that the lipidated form of E6mE7m is able to activate dendritic cells and trigger innate immunity.

### *Enhancement of antigen-specific antibody levels and Th1 responses by immunization with ripo-E6mE7m*

Next, we examined lymphocyte proliferation in response to rE6mE7m and ripo-E6mE7m in



**Figure 4.** Immunization with rlipo-E6mE7m induced higher levels of T cell proliferation than immunization with rE6mE7m. (A) Lymphocytes from the spleen were isolated from protein-immunized C57BL/6 mice and plated at a density of  $2 \times 10^5$  cells/well in 96-well plates. The cells were incubated with 10  $\mu$ g/ml rE6mE7m and rlipo-E6mE7m for 48 hrs. In the final 24 hrs, 1  $\mu$ Ci [ $^3$ H]-thymidine was added to each well to measure DNA synthesis. The data are represented as the mean  $\pm$  SD of triplicate samples. (B) The immunization with rlipo-E6mE7m induced higher levels of cytokine release than the immunization with rE6mE7m. Mice were injected subcutaneously with 10  $\mu$ g rE6mE7m or rlipo-E6mE7m twice at two-week intervals. Seven days after the second immunization, the splenocytes were isolated and stimulated with rE6mE7m or rlipo-E6mE7m (10  $\mu$ g/ml) for 4-5 days. The supernatants were collected, and the levels of IFN- $\gamma$ , IL-5, IL-10 or IL-17A were measured by ELISAs. The data are expressed as the mean  $\pm$  SD of the samples (n=3).

cells from mice treated with immunogen immunization using [ $^3$ H]-thymidine to measure DNA synthesis. Both immunogens stimulated the proliferation of splenocytes, but rlipo-E6mE7m induced higher proliferation than rE6mE7m (Figure 4A). In addition, the secretion of IFN- $\gamma$ , IL-5, IL-10, and IL-17A by T helper cells isolated from immunogen-immunized mice was increased by stimulation with either of the two immunogens, and rlipo-E6mE7m induced higher levels of cytokine secretion than the rE6mE7m (Figure 4B). Thus, the results demonstrated that the lipidated immunogen has better adjuvant properties than the nonlipid immunogen, which allows the lipidated immunogen to acti-

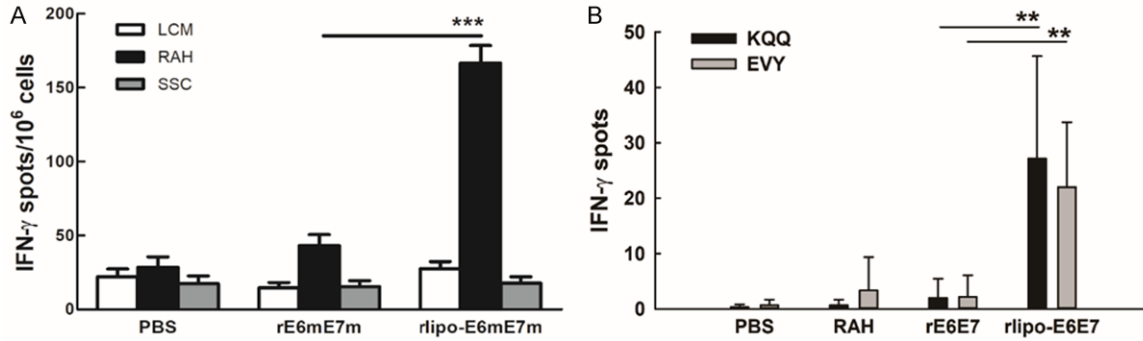
vate T helper cell proliferation and cytokine secretion.

#### *Immunization with rlipo-E6mE7m induces E6- and E7-specific T cell responses*

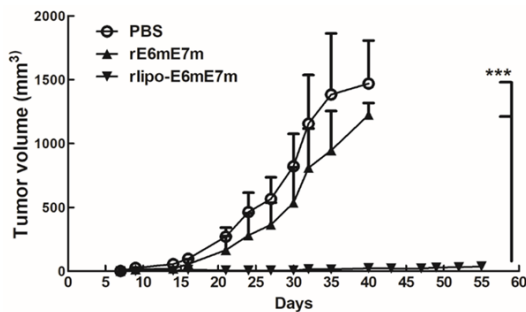
We then investigated whether rlipo-E6mE7m immunization can induce E7-specific CTL responses. After C57BL/6 mice were subcutaneously immunized twice (day 0 and 7) with the immunogens, the splenocytes from immunized mice were harvested 7 days after the last boost. Since the RAHYNIVTF peptide (RAH) of HPV16 E7 is a known H-2D<sup>b</sup>-restricted CTL epitope, the RAH peptide was used to stimulate the cultured splenocytes to assess the E7-specific CTL responses using an IFN- $\gamma$  ELISpot assay (see Materials and Methods). The mice immunized with rlipo-E6mE7m exhibited a higher number of IFN- $\gamma$  secreting cells than those immunized with rE6mE7m (Figure 5A). Furthermore, to investigate the E6-specific T cell response, the EVY peptides of HPV E6 that have been identified as H-2K<sup>b</sup>-restricted CTL epitopes were used for specificity evaluation [23]. With the stimulation by the HPV E6 peptides KQQ (longer peptides containing EVY sequence) and EVY, more IFN- $\gamma$  secreting cells were activated in the rlipo-E6mE7m immunized groups than in the rE6mE7m groups (Figure 5B). These results clearly demonstrated that the HPV E6- and E7-specific CTL responses can be induced using rlipo-E6mE7m vaccination.

#### *Evaluation of the therapeutic effect of the rlipo-E6mE7m immunogens*

To evaluate the antitumor efficacy of the immunogens *in vivo*, mouse lung cancer TC-1 cells, which were transformed with the E6 and E7



**Figure 5.** Immunization with rliipo-E6mE7m induced higher levels of E7-specific IFN- $\gamma$ -secreting cells than the immunization with rE6mE7m. (A) Splenocytes or lymph node cells ( $2 \times 10^5$  cells/well) from immunized mice were incubated with lymphocyte culture medium control (LCM), 10 mg/ml RAHYNIVTF (RAH) peptide, or irrelevant peptide (SSC) for 48 hrs in an anti-IFN- $\gamma$ -coated 96-well ELISPOT plate. The IFN- $\gamma$ -secretion spots were measured using an ELISPOT reader. The data are expressed as the mean  $\pm$  SD of three animals per group. (B) The immunization with rliipo-E6mE7m induces E6-specific IFN- $\gamma$  secretion. Splenocytes ( $2 \times 10^5$  cells/well) from immunized mice were incubated with or without 10 mg/ml HPV16 E6 peptides KQQLLRREYDFAFRDLICIVYRDGN (KQQ) or EYDFAFRDL (EVY) for 48 hrs in an anti-IFN- $\gamma$  antibody-coated 96-well ELISPOT plate. The IFN- $\gamma$ -secretion spots were measured using an ELISPOT reader. The data are expressed as the mean  $\pm$  SD of three animals per group.



**Figure 6.** Treatment with rliipo-E6mE7m at day 7 after tumor inoculation inhibited tumor growth, but treatment with rE6mE7m did not. Mice were subcutaneously inoculated with TC-1 cells ( $2 \times 10^5$ /mouse). After 7 days, the tumor-bearing mice (5-10 mice per group) were injected once with RAH/IFA (10  $\mu$ g/mouse), rE6mE7m (30  $\mu$ g/mouse), rliipo-E6mE7m (30  $\mu$ g/mouse) or PBS. The tumor volumes were calculated as length  $\times$  width  $\times$  width/2 (mm<sup>3</sup>). The data are expressed as the mean  $\pm$  SEM.

genes, were subcutaneously injected into C57BL/6 mice. Seven days later, these mice were subcutaneously injected with 30  $\mu$ g rE6mE7m or rliipo-E6mE7m in the abdomen. In the PBS or rE6mE7m treatment, the tumors grew quickly within one month (Figure 6). However, the tumors were scarcely detected after day 55 in the mice receiving rliipo-E6mE7m. The results demonstrated that the therapeutic efficacy of the recombinant immunogens was dramatically increased in the lipidated form.

## Discussion

In our previous studies, rliipo-E7m was shown to induce Th1 immune responses, E7-specific CTL responses, and tumor-protective immunity in a mouse tumor model [21]. We further demonstrated that rliipo-E7m combined with the TLR9 agonist CpG ODN not only inhibited tumor growth but also reduced the number of immunosuppressive cells in the tumor microenvironment [24]. Interestingly, the therapeutic effects of rliipo-E7m/CpG can be enhanced when rliipo-E7m/CpG is combined with the anticancer drug gemcitabine. Moreover, the combination therapy can reduce the number of PD-1-positive E7-specific T lymphocytes [25]. Despite this result, E6 still needs to be incorporated into the formulation of a therapeutic vaccine. In particular, E6 can bind to p53 to induce the degradation of p53, which plays an important role in cell transformation.

In this study, we designed a lipidated fusion protein that contained mutant E6 and E7 (rliipo-E6mE7m), in which p53 and pRb binding activity are lost, respectively. The nonlipidated recombinant E6mE7m (rE6mE7m) was produced for comparison. HPV16 E6E7 fusion proteins have been designed for therapeutic vaccines using different approaches [26-30]. Because the protein-based vaccine has a high safety profile, it would be easier to fit into the regulatory requirement. However, the challeng-



## A recombinant lipidated HPV E6 and E7 fusion protein induces antitumor immunity

es of developing a protein-based therapeutic vaccine against tumors are low immunogenicity and difficulty in eliciting CTL responses. To overcome these limitations, the delivery of the antigen to antigen presenting cells in a way that activates the antigen presenting cells is key. The lipidated antigen rliipo-E6mE7m can effectively target antigen presenting cells through TLR2 and induce robust CTL responses.

In fact, the recombinant E6E7 fusion protein was a highly immunogenic protein that induced T cell responses in the absence of an adjuvant (**Figure 4**). However, the number of the CTL responses induced by recombinant E6mE7m was still lower than that of those induced by rliipo-E6mE7m (**Figure 5**). The induction of CTL responses in the rliipo-E6mE7m group may have occurred due to the increasing cross-presentation of antigenic peptides to CD8<sup>+</sup> T cells mediated by TLR2 signaling. Our previous study showed that the TLR2-conjugated peptide can be taken up by dendritic cells and activate the MyD88-dependent signaling pathway to increase the cross-presentation of the peptide [31]. A nonadjuvanted HPV E6E7 multiepitope vaccine that can induce antitumor effects after three doses of immunization has also been developed [27]. Similarly, we observed that rE6mE7m alone can induce antigen-specific T cell responses, but it induces fewer CTL responses than rliipo-E6mE7m. These results demonstrated that the induction of efficient CTL responses requires efficient antigen targeting to antigen presenting cells and the activation of antigen presenting cells. The targeting and activation of antigen presenting cells could occur through TLR signaling or through other innate receptors. Here, we used lipidating technology to generate rliipo-E6mE7m, which has TLR2 agonist activity to efficiently induce antitumor immunity. The technology can be applied to different tumor-associated antigens to generate therapeutic cancer vaccines for different cancers.

### Acknowledgements

This work was supported by a Research Investigator Award from the National Health Research Institutes (IV-106-PP-17 to S.-J. L.) in Taiwan and Chinese Medicine Research Center, China Medical University from The Featured Areas Research Center Program within the

framework of the Higher Education Sprout Project by the Ministry of Education in Taiwan (CMRC-CHM-8).

### Disclosure of conflict of interest

None.

**Address correspondence to:** Shih-Jen Liu and Chih-Hsiang Leng, National Institute of Infectious Diseases and Vaccinology, National Health Research Institute, 35 Keyan Road, Zhunan, Miaoli 350, Taiwan, R.O.C. Tel: 886-3-724-6166 Ext. 37709; Fax: 886-3-758-3009; E-mail: levent@nhri.org.tw (SJL); leoleng@nhri.org.tw (CHL)

### References

- [1] Schwarz TF. AS04-adjuvanted human papillomavirus-16/18 vaccination: recent advances in cervical cancer prevention. *Expert Rev Vaccines* 2008; 7: 1465-1473.
- [2] Ferlay J, Shin HR, Bray F, Forman D, Mathers C and Parkin DM. Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. *Int J Cancer* 2010; 127: 2893-2917.
- [3] Harper DM. Currently approved prophylactic HPV vaccines. *Expert Rev Vaccines* 2009; 8: 1663-1679.
- [4] Tan S, de Vries EG, van der Zee AG and de Jong S. Anticancer drugs aimed at E6 and E7 activity in HPV-positive cervical cancer. *Curr Cancer Drug Targets* 2012; 12: 170-184.
- [5] Hoppe-Seyler K, Bossler F, Braun JA, Herrmann AL and Hoppe-Seyler F. The HPV E6/E7 oncogenes: key factors for viral carcinogenesis and therapeutic targets. *Trends Microbiol* 2018; 26: 158-168.
- [6] Vande Pol SB and Klingelhutz AJ. Papillomavirus E6 oncoproteins. *Virology* 2013; 445: 115-137.
- [7] Riley RR, Duensing S, Brake T, Munger K, Lambert PF and Arbeit JM. Dissection of human papillomavirus E6 and E7 function in transgenic mouse models of cervical carcinogenesis. *Cancer Res* 2003; 63: 4862-4871.
- [8] Trimble CL and Frazer IH. Development of therapeutic HPV vaccines. *Lancet Oncol* 2009; 10: 975-980.
- [9] zur Hausen H. Papillomaviruses and cancer: from basic studies to clinical application. *Nat Rev Cancer* 2002; 2: 342-350.
- [10] Yang A, Farmer E, Wu TC and Hung CF. Perspectives for therapeutic HPV vaccine development. *J Biomed Sci* 2016; 23: 75.
- [11] Kovacs-Simon A, Titball RW and Michell SL. Lipoproteins of bacterial pathogens. *Infect Immun* 2011; 79: 548-561.

## A recombinant lipidated HPV E6 and E7 fusion protein induces antitumor immunity

- [12] Christodoulides A, Boyadjian A and Kelesidis T. Spirochetal lipoproteins and immune evasion. *Front Immunol* 2017; 8: 364.
- [13] Thanassi WT and Schoen RT. The Lyme disease vaccine: conception, development, and implementation. *Ann Intern Med* 2000; 132: 661-668.
- [14] Rau H, Revets H, Cornelis P, Titzmann A, Ruggli N, McCullough KC and Summerfield A. Efficacy and functionality of lipoprotein Oprl from *Pseudomonas aeruginosa* as adjuvant for a subunit vaccine against classical swine fever. *Vaccine* 2006; 24: 4757-4768.
- [15] Wilkinson KA, Newton SM, Stewart GR, Martineau AR, Patel J, Sullivan SM, Herrmann JL, Neyrolles O, Young DB and Wilkinson RJ. Genetic determination of the effect of post-translational modification on the innate immune response to the 19 kDa lipoprotein of *Mycobacterium tuberculosis*. *BMC Microbiol* 2009; 9: 93.
- [16] Chen HW, Liu SJ, Liu HH, Kwok Y, Lin CL, Lin LH, Chen MY, Tsai JP, Chang LS, Chiu FF, Lai LW, Lian WC, Yang CY, Hsieh SY, Chong P and Leng CH. A novel technology for the production of a heterologous lipoprotein immunogen in high yield has implications for the field of vaccine design. *Vaccine* 2009; 27: 1400-1409.
- [17] Infante-Duarte C and Kamradt T. Lipopeptides of *Borrelia burgdorferi* outer surface proteins induce Th1 phenotype development in alpha-beta T-cell receptor transgenic mice. *Infect Immun* 1997; 65: 4094-4099.
- [18] Oftung F, Wiker HG, Deggerdal A and Mustafa AS. A novel mycobacterial antigen relevant to cellular immunity belongs to a family of secreted lipoproteins. *Scand J Immunol* 1997; 46: 445-451.
- [19] Shai A, Brake T, Somoza C and Lambert PF. The human papillomavirus E6 oncogene dysregulates the cell cycle and contributes to cervical carcinogenesis through two independent activities. *Cancer Res* 2007; 67: 1626-1635.
- [20] Strati K and Lambert PF. Role of Rb-dependent and Rb-independent functions of papillomavirus E7 oncogene in head and neck cancer. *Cancer Res* 2007; 67: 11585-11593.
- [21] Huang CY, Chen JJ, Shen KY, Chang LS, Yeh YC, Chen IH, Chong P, Liu SJ and Leng CH. Recombinant lipidated HPV E7 induces a Th-1-biased immune response and protective immunity against cervical cancer in a mouse model. *PLoS One* 2012; 7: e40970.
- [22] Cooper B, Schneider S, Bohl J, Jiang Y, Beaudet A and Vande Pol S. Requirement of E6AP and the features of human papillomavirus E6 necessary to support degradation of p53. *Virology* 2003; 306: 87-99.
- [23] Stauss HJ, Davies H, Sadovnikova E, Chain B, Horowitz N and Sinclair C. Induction of cytotoxic T lymphocytes with peptides in vitro: identification of candidate T-cell epitopes in human papilloma virus. *Proc Natl Acad Sci U S A* 1992; 89: 7871-7875.
- [24] Chang LS, Leng CH, Yeh YC, Wu CC, Chen HW, Huang HM and Liu SJ. Toll-like receptor 9 agonist enhances anti-tumor immunity and inhibits tumor-associated immunosuppressive cells numbers in a mouse cervical cancer model following recombinant lipoprotein therapy. *Mol Cancer* 2014; 13: 60.
- [25] Chang LS, Yan WL, Chang YW, Yeh YC, Chen HW, Leng CH and Liu SJ. Gemcitabine enhances antitumor efficacy of recombinant lipoid-munogen-based immunotherapy. *Oncoimmunology* 2016; 5: e1095433.
- [26] Almajhdi FN, Senger T, Amer HM, Gissmann L and Ohlschlager P. Design of a highly effective therapeutic HPV16 E6/E7-specific DNA vaccine: optimization by different ways of sequence rearrangements (shuffling). *PLoS One* 2014; 9: e113461.
- [27] de Oliveira LM, Morale MG, Chaves AA, Cavalher AM, Lopes AS, Diniz Mde O, Schanoski AS, de Melo RL, Ferreira LC, de Oliveira ML, Demasi M and Ho PL. Design, immune responses and anti-tumor potential of an HPV16 E6E7 multi-epitope vaccine. *PLoS One* 2015; 10: e0138686.
- [28] Peng S, Wang JW, Karanam B, Wang C, Huh WK, Alvarez RD, Pai SI, Hung CF, Wu TC and Roden RB. Sequential cisplatin therapy and vaccination with HPV16 E6E7L2 fusion protein in saponin adjuvant GPI-0100 for the treatment of a model HPV16+ cancer. *PLoS One* 2015; 10: e116389.
- [29] Garcia-Bates TM, Kim E, Concha-Benavente F, Trivedi S, Mailliard RB, Gambotto A and Ferris RL. Enhanced cytotoxic CD8 T cell priming using dendritic cell-expressing human papillomavirus-16 E6/E7-p16INK4 fusion protein with sequenced anti-programmed death-1. *J Immunol* 2016; 196: 2870-2878.
- [30] Atherton MJ, Stephenson KB, Nikota JK, Hu QN, Nguyen A, Wan Y and Lichty BD. Preclinical development of peptide vaccination combined with oncolytic MG1-E6E7 for HPV-associated cancer. *Vaccine* 2018; 36: 2181-2192.
- [31] Shen KY, Song YC, Chen IH, Leng CH, Chen HW, Li HJ, Chong P and Liu SJ. Molecular mechanisms of TLR2-mediated antigen cross-presentation in dendritic cells. *J Immunol* 2014; 192: 4233-4241.